Practitioner's Docket No.: 802_003 ***PATENT***

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Patrick T. PRENDERGAST

Ser. No.: 10/091,855 Art Unit: 1617

Filed: March 6, 2002 Examiner: Theodore J. Criares

Conf. No.: 8597

For: COMBINATION THERAPY FOR REDUCTION OF TOXICITY OF CHEMOTHERAPEUTIC AGENTS

Commissioner for Patents
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Janet M. Stevens

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RESPONSE TO REQUIREMENT FOR ELECTION OF SPECIES

Sir:

The following elections are made in response to the Office Action mailed May 21, 2004.

The May 21, 2004 Office Action includes the statement "Applicant's election with traverse of the claims of Group Alzheimer's disease (claims 18-44 in Paper dated December 23, 2003 is acknowledged." It is respectfully noted that the claims in the elected group (i.e., group II, claims 18-44) are directed to a method of treating a patient suffering from neoplasia, not Alzheimer's disease.

The May 21, 2004 Office Action contains a requirement that the Applicant "elect a single disclosed species (combination of a active agents to treat a single cancer listed in claims 31 or 31)." In response, the Applicant elects the combination of circiliol as the compound of claim 18 and gemcitabine as the chemotherapeutic agent. In addition, the

Applicant elects pancreatic cancer as the type of neoplasia recited in claims 30 and 31. In addition, it is respectfully requested that the search and examination of the present application also be carried out with respect to lung cancer. It is respectfully submitted that including lung cancer, as well as the elected pancreatic cancer, would not impose an undue burden on the U.S. PTO.

The May 21, 2004 Office Action also includes a request that the Applicant "identify the active agent 'circiliol' by chemical name, structure and if it was publicly available as of the filing date of the priority application." In response, it is respectfully noted that as of the filing date of the present application, it was well known to those of skill in the art that circiliol is 5,3',4'-trihydroxy-6,7 dimethoxy flavone, the structure of which is shown on the attached sheet. In addition, attached is a copy of an article entitled "Effects of Cirsiliol, a Flavone Isolated from *Achillea Fragrantissima*, on rat isolated ileum," dated 22 July 1991, confirming the chemical name for circiliol (see page 555, left column, lines 9-10).

The U.S. PTO is reminded that upon reaching a conclusion that the elected subject matter is allowable, the search and examination of this application should be continued to the non-elected species.

If the Examiner believes that contact with Applicant's attorney would be advantageous toward the disposition of this case, the Examiner is herein requested to call Applicant's attorney at the phone number noted below.

The Commissioner is hereby authorized to charge any additional fees associated with this communication or credit any overpayment to Deposit Account No. 50-1446.

Respectfully submitted,

November 19, 2004
Date

Kevin C. Brown
Reg. No. 32,402

KCB:jms
Enclosures:
Article entitled "Effects of Cirsiliol, a Flavone Isolated from *Achillea Fragrantissima*, on rat isolated ileum," dated 22 July 1991
Sheet showing the chemical structure of circiliol

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Syracuse, NY 13261-7068

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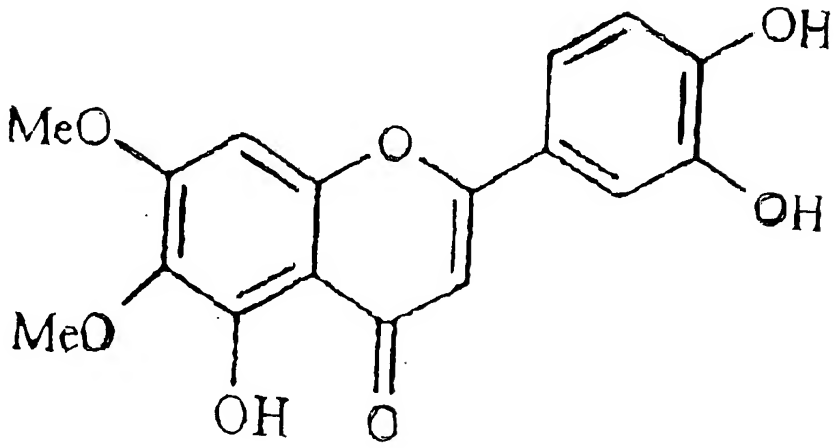

(12) Circiliol

AD

EFFECTS OF CIRSILIOL, A FLAVONE ISOLATED FROM *ACHILLEA FRAGRANTISSIMA*, ON RAT ISOLATED ILEUM

E. H. MUSTAFA,[1] M. ABU ZARGA[2] and S. ABDALLA[1]*

[1]Department of Biological Sciences and [2]Department of Chemistry, Faculty of Science, University of Jordan, Amman, Jordan

(Received 22 July 1991)

Abstract—1. In concentrations from 10^{-8} M to 3×10^{-4} M, cirsiliol caused concentration-dependent relaxation of rat isolated ileum.

2. Phentolamine (10^{-6} M) or phentolamine and propranolol (10^{-6} M) had no significant effects on the concentration–effect curves or on the EC_{50} of cirsiliol on the ileum.

3. Cirsiliol shifted to the right the acetylcholine (Ach) concentration–effect curves on ileum and significantly inhibited the maximum contractions induced by Ach.

4. In Ca^{2+}-free, depolarizing solution, cirsiliol shifted to the right the $CaCl_2$ concentration–effect curves and inhibited the maximum contractions induced by $CaCl_2$ on ileum.

5. Large concentrations (10^{-4} M, 3×10^{-4} M) of cirsiliol induced relaxation followed by contraction of the ileal segments incubated in Ca^{2+}-free solution.

6. In Ca^{2+}-free solution, cirsiliol (10^{-4} M, 3×10^{-4} M) caused concentration-dependent potentiation of the ileal contractions induced by 3×10^{-3} M Ach when the latter was added 2–3 min after cirsiliol. When Ach was added 15–20 min after cirsiliol, the latter compound inhibited the Ach-induced contractions.

7. These observations suggest that cirsiliol inhibits Ca^{2+} influx but stimulates Ca^{2+} release from intracellular stores. Furthermore, they suggest that cirsiliol utilizes the same Ca^{2+} source used by acetylcholine in Ca^{2+}-free solution.

INTRODUCTION

Achillea fragrantissima has been reputed in folk medicine in the Arabia region for the treatment of gastrointestinal disturbances (Segal *et al.*, 1987). Although previous studies were carried out on this species, a thorough phytochemical and pharmacological investigation has not been performed. In an attempt to identify the ingredients responsible for the spasmolytic activity, we have been able to isolate the flavone aglycone cirsiliol (5,3′,4′-trihydroxy-6,7-dimethoxyflavone). Flavonoids have been the subject of extensive study for many years (Kyriakidis *et al.*, 1986) and they possess a wide spectrum of physiological and pharmacological effects (Griffiths, 1982; Yoshimoto *et al.*, 1983; Macander, 1986). Although cirsiliol has been isolated and identified previously from many plant species (Mues *et al.*, 1979), to the best of our knowledge, no studies on its biological effects have been carried out. This study was undertaken to evaluate the effects of cirsiliol on isolated ileal smooth muscle from the rat. The results point to the possible interference by cirsiliol with Ca^{2+} metabolism in ileal smooth muscle.

MATERIALS AND METHODS

Animals and preparations

Male and female albino rats (200–300 g) were sacrificed by a sharp blow to the back of the head. The abdomen was opened to obtain the ileal longitudinal segments. The tissues were removed and prepared for recording of isometric contraction as described previously (Abdalla and Abu Zarga, 1987). Recording was performed using Grass force–displacement transducer (FTO3C) connected to a Gilson polygraph.

Protocol of the experiments

After 2 hr equilibration in the tissue bath, concentration–effect curves of cirsiliol on the ileal longitudinal segments were performed. Since cirsiliol was dissolved in 0.1 N NaOH, the control tissues were treated with the proper concentration of the solvent (0.1 N NaOH in 0.9% NaCl; final bath concentration of NaOH was 0.3% v/v). When the experiment was terminated, preparations were blotted with filter paper and weighed and the responses of tissues were expressed as g/g tissue. In a second series of experiments, concentration–effect curves of cirsiliol on the ileum were established in the absence and the presence of phentolamine (10^{-6} M) or phentolamine and propranolol (10^{-6} M). In a third series of experiments, concentration–effect curves of Ach (10^{-6}–3×10^{-3} M) on the ileum were established in the absence and the presence of various concentrations of cirsiliol (3×10^{-5}, 10^{-4}, 3×10^{-4} M). Cirsiliol was added 20 min before Ach and remained in the tissue bath all through the experiment. In a fourth set of experiments, the physiological salt solution (PSS) was replaced by a nominally Ca^{2+}-free, depolarizing solution and tissues were incubated in this solution for 30 min and $CaCl_2$ concentration–effect curves in the absence and the presence of various concentrations of cirsiliol were then established. Tissues were exposed to cirsiliol for 30 min then $CaCl_2$ was added cumulatively to a concentration of 3×10^{-2} M. In another set of experiments, single doses (3×10^{-5}, 10^{-4}, 3×10^{-4} M) of cirsiliol were added to the

*To whom all correspondence should be addressed.

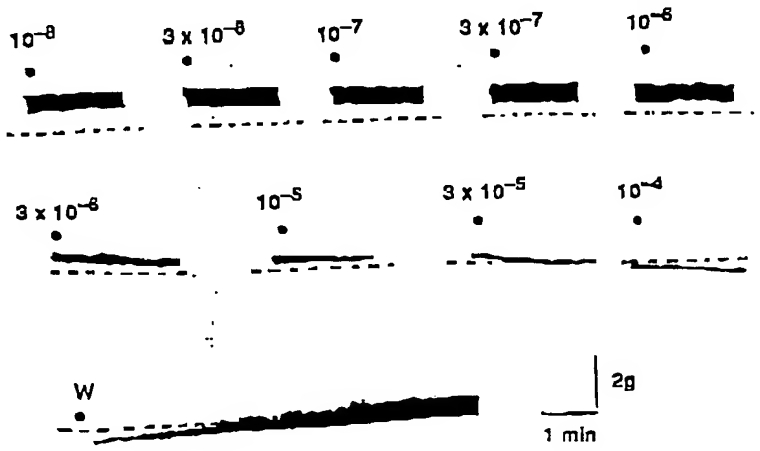

Fig. 1. Typical responses of rat isolated ileum to increasing concentrations (mole/liter) of cirsiliol. W represents removal of the compound from the tissue bath.

ileum that had been incubated for 5 min in Ca^{2+}-free solution and the responses of the tissues were noted. In yet another series of experiments, the tissues were incubated in Ca^{2+}-free solution for 25 min then treated with an agonist, acetylcholine (Ach), to obtain the maximum contractions. Tissues were then washed with PSS for 1 hr and incubated again with Ca^{2+}-free solution for another 25 min. Tissues were treated then with various concentrations of cirsiliol and Ach (3×10^{-3} M) was added again to record the maximum contractions in the presence of cirsiliol. Ach was added 2–3 min or 15–20 min after cirsiliol. The contractile responses of each tissue to the agonist (Ach) in the absence and the presence of cirsiliol were expressed as g/g tissue and compared.

Solutions

Physiological salt solution (PSS). This was prepared daily and had the following composition in mM: NaCl 118, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 2.5, $MgCl_2 \cdot 6H_2O$ 0.5, NaH_2PO_4 1.0, $NaHCO_3$ 25.0 and dextrose 11.1. The Ca^{2+}-free, depolarizing solution had a similar composition to PSS except that $CaCl_2$ and NaCl had been removed and that KCl was increased to a concentration of 122.7 mM. Acetylcholine chloride (BDH chemicals Ltd) and propranolol (Sigma) were dissolved in 0.9% NaCl. Phentolamine hydrochloride or phentolamine HCl was dissolved in distilled water.

Cirsiliol solution. The compound was isolated from *Achillea fragrentissima* and was identified by direct comparison of its UV, MS, [^{1}H]NMR and mp with literature data (Mues *et al.*, 1979). Cirsiliol was dissolved in minimal volume of 0.1 N NaOH and completion of the volume was made with 0.9% NaCl. Stock solution of cirsiliol was wrapped with aluminum foil to protect

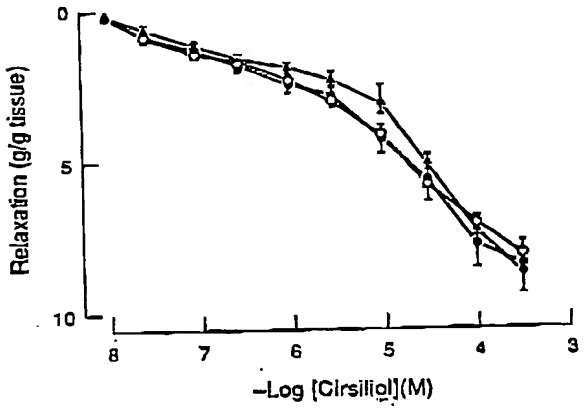

Fig. 2. Concentration–effect curves of cirsiliol on rat isolated ileum in the absence (solid triangles) and the presence of phentolamine (solid circles) or phentolamine and propranolol (open circles). Vertical bars represent SEM of six experiments.

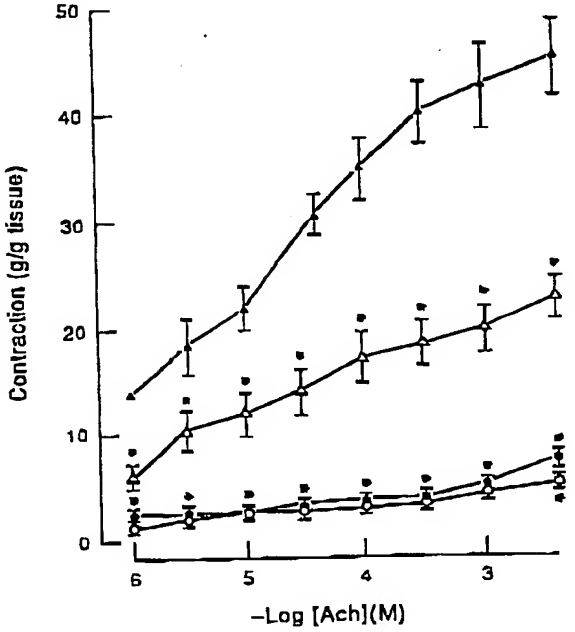

Fig. 3. Concentration–effect curves of acetylcholine on rat ileum in the absence (solid triangles) and the presence of cirsiliol: 3×10^{-5} M (open triangles), 10^{-4} M (closed circles) or 3×10^{-4} M (open circles).

Table 1. Effects of various concentrations of cirsiliol on the maximum contractions induced in rat isolated ileum by acetylcholine or by $CaCl_2$ and on their EC_{50} values*

Treatment	Acetylcholine		$CaCl_2$	
	EC_{50} (M)	Max. contraction (g/g)	EC_{50} (M)	Max. contraction (g/g)
Control	$(5.0 \pm 1.4)10^{-6}$	45.0 ± 3.0	$(4.1 \pm 0.7)10^{-4}$	15.6 ± 1.9
Cirsiliol 3×10^{-5} M	$(2.3 \pm 0.5)10^{-3}$†	23.2 ± 2.4†	$(1.0 \pm 0.4)10^{-2}$†	10.9 ± 1.1†
Cirsiliol 1×10^{-4} M	$>3 \times 10^{-3}$	7.9 ± 1.2†	$(3.0 \pm 0.9)10^{-2}$†	7.7 ± 1.4†
Cirsiliol 3×10^{-4} M	$>3 \times 10^{-3}$	5.2 ± 1.1†	$>3 \times 10^{-2}$	0.0 ± 0.0†

*All values are means ± SEM of six experiments.
†$P < 0.05$ compared to the control; t-test for independent samples.

against photooxidation. Stock solutions were kept refrigerated until shortly before use and dilutions thereof were made in 0.9% NaCl daily.

Statistical analysis

Data are presented as means ± SEM. The values of EC_{50} in the absence and the presence of cirsiliol were compared by Student t-test for either paired or independent samples. Comparison of the concentration–effect curves in the absence and the presence of cirsiliol was performed at each level of agonist using Student t-test for independent samples. Differences were considered significant when P was less than 0.5.

RESULTS

Figures 1 and 2 show that cirsiliol, in concentrations from 10^{-8}–3×10^{-4} M, caused concentration-dependent inhibition of the amplitude of the phasic contractions and relaxed the tone of the ileal segments. The effect of cirsiliol on this tissue was reversible upon the removal of the compound from the tissue bath (Fig. 1). The EC_{50} of cirsiliol for relaxation of ileal segments was $(5.2 \pm 0.9) \times 10^{-6}$ M ($n = 6$). The solvent (0.1 N NaOH in 0.9% NaCl) had no observed effect on the tone or on the contractions of the ileum and it was not tested any further accordingly. Phentolamine (10^{-6} M) or phentolamine and propranolol (10^{-6} M) had no significant effects on cirsiliol concentration–effect curve on the ileum (Fig. 2). The EC_{50} of cirsiliol in the presence of phentolamine or phentolamine and propranolol were $(5.5 \pm 0.4)10^{-6}$, $(5.2 \pm 0.7)10^{-6}$ M respectively.

In concentrations of 3×10^{-5}, 10^{-4}, 3×10^{-4} M cirsiliol caused a rightward shift of the concentration–effect curve of acetylcholine on the ileum (Fig. 3). Cirsiliol significantly reduced the maximum contractions induced by 3×10^{-3} M Ach on the ileum and increased the EC_{50} of Ach on this tissue (Table 1).

To determine the possible role of cirsiliol on Ca^{2+} influx and/or binding to Ca^{2+}-binding proteins, ileal segments were exposed to cirsiliol in a nominally Ca^{2+}-free depolarizing solution and Ca^{2+} was added exogenously to establish $CaCl_2$ concentration–effect curves. Cirsiliol (3×10^{-5}, 10^{-4}, 3×10^{-4} M) induced concentration-dependent rightward shifts of $CaCl_2$ concentration–effect curves and caused concentration-dependent inhibition of the maximum contraction induced by 3×10^{-2} M $CaCl_2$ (Fig. 4). Effects of cirsiliol on the maximum contraction and on the EC_{50} of $CaCl_2$ on these preparations are shown in Table 1.

Addition of cirsiliol (10^{-4}, 3×10^{-4} M) to the ileal segments incubated in Ca^{2+}-free solution showed that cirsiliol induced slight relaxation followed by contraction within 2–15 min (Fig. 5I). Cirsiliol (10^{-4} M) caused a contraction that amounts to 1.39 ± 0.24 g/g tissue ($n = 6$) while 3×10^{-4} M cirsiliol caused a contraction that amounts to 3.90 ± 0.32 g/g tissue on the ileal segments incubated in Ca^{2+}-free solution.

In order to determine the possible role of cirsiliol on Ca^{2+} release from intracellular stores, ileal segments were exposed to a Ca^{2+}-free solution. When these tissues were challenged with 3×10^{-3} M Ach they responded by transient contractions. 3×10^{-5} M cirsiliol had no significant effect on the transient contractions induced by Ach, while larger concentrations (10^{-4} M, 3×10^{-4} M) caused an increase in the maximum phasic contractions induced by Ach when the latter was added 2–3 min after cirsiliol (Fig. 5IIB). The effects of cirsiliol on Ach-induced contractions are shown in Table 2 and Fig. 5. The addition of Ach to the ileal segments 15–20 min after the addition of cirsiliol caused significant inhibition of the maximum contractions induced by Ach

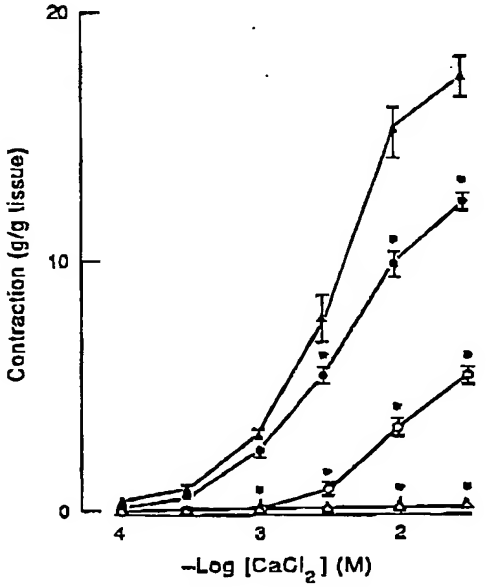

Fig. 4. Concentration–effect curves of $CaCl_2$ on rat ileum in the absence (solid triangles) and the presence of cirsiliol: 3×10^{-5} M (solid circles), 10^{-4} M (open circles) or 3×10^{-4} M (open triangles).

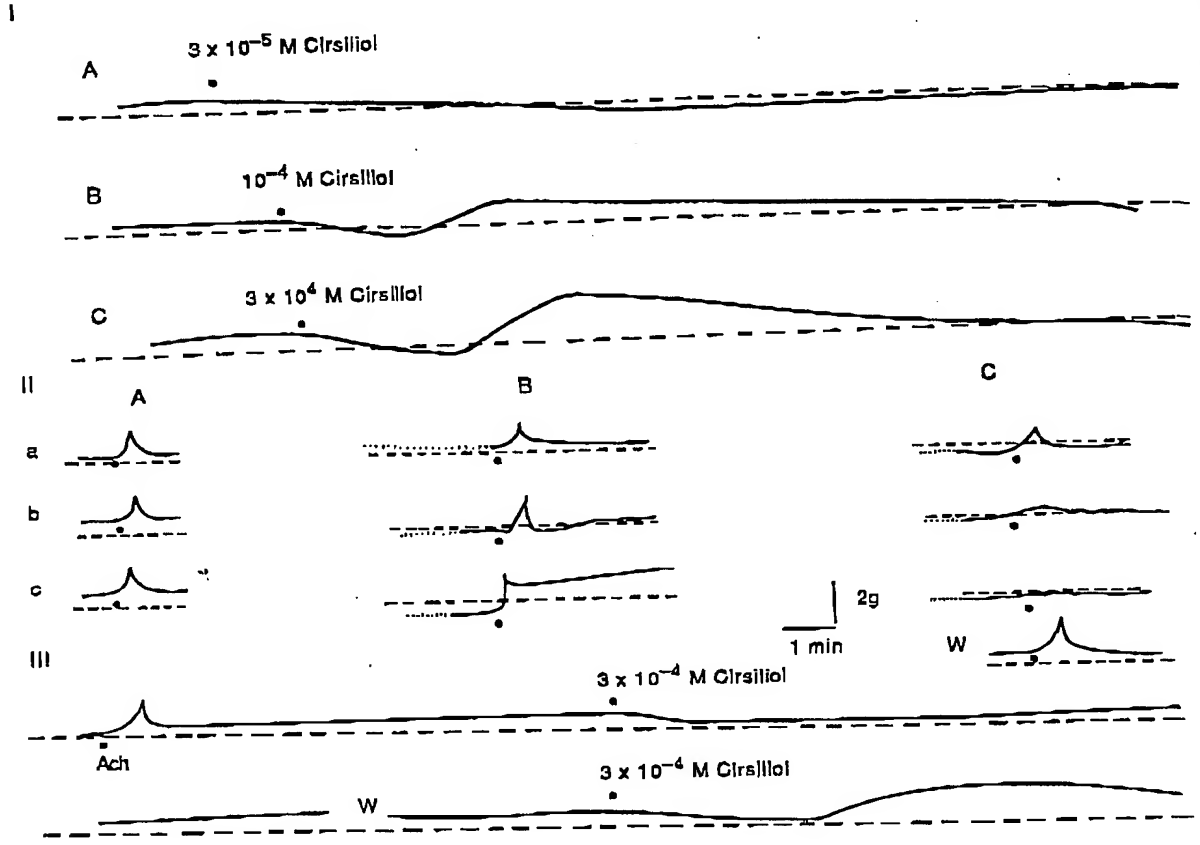

Fig. 5. I. Effects of increasing concentrations of cirsiliol on rat ileum incubated in Ca^{2+}-free solution. II. Responses of ileum in Ca^{2+}-free solution to the addition of 3×10^{-3} M acetylcholine (●) in the absence (A) or the presence (B, C) of various concentrations of cirsiliol: 3×10^{-5} M (a), 10^{-4} M (b) or 3×10^{-4} M (c). B: responses when acetylcholine was added 2–3 min after cirsiliol. C: when added 15–20 min after cirsiliol. III. Effect on ileum of the addition of cirsiliol after acetylcholine (Ach) in Ca^{2+}-free solution and after reincubation in Ca^{2+}-containing solution followed by Ca^{2+}-free solution added at W.

(Fig. 5IIC). When the ileal segments were washed with PSS for 1 hr, incubated again in Ca^{2+}-free solution and treated with Ach the maximum contractions in response to Ach were restored (Fig. 5IIC).

On another set of experiments ileal segments were incubated in Ca^{2+}-free solution for 25 min and treated with 3×10^{-3} M Ach. It was found that addition of 3×10^{-4} M cirsiliol under these conditions slightly relaxed the tone of the ileal segments while no contraction appeared (Fig. 5III). When the ileal segments were washed with PSS for 1 hr, incubated again in Ca^{2+}-free solution and treated with 3×10^{-4} M cirsiliol, it was found that the contractions in response to cirsiliol were restored (Fig. 5III).

Table 2. Effects of cirsiliol on the maximum contractions induced by 3×10^{-3} M Ach on rat isolated ileum incubated in Ca^{2+}-free solution*

	Maximum contraction (g/g tissue)		% potentiation
	Control	Treated	
Cirsiliol 1×10^{-4} M	2.9 ± 0.36	4.4 ± 0.83†	28.5 ± 7.8%
Cirsiliol 3×10^{-4} M	3.8 ± 0.68	4.6 ± 1.30†	37.3 ± 6.3%

*All values are means ± SEM of six experiments.
†$P < 0.05$ compared to the control experiments; paired t-test.

DISCUSSION

The present experiments demonstrate that cirsiliol has an inhibitory effect on rat isolated ileal segments. Since the action of cirsiliol on these segments was rapid and reversible, it is unlikely that this effect is due to the impairment of energy production or due to a permanent change of the receptor molecule. Also, since treatments with phentolamine or with phentolamine and propranolol did not affect the position, or the maximum relaxation, of the concentration–effect curves of cirsiliol on rat isolated ileum, it is unlikely that this relaxation is mediated by α or β adrenergic receptors. Stimulation of these two receptor types is known to mediate relaxation of gastrointestinal smooth muscle of several mammalian species (Fagbemi and Salako, 1980; Fontaine *et al.*, 1984; Demol *et al.*, 1989).

In smooth muscle cells, as well as in cardiac and skeletal muscle cells, it has been assumed that changes in force development are directly related to changes in the cytoplasmic Ca^{2+} concentration [Ca^{2+}] (Nayler and Grinwald, 1981; Casteels and Droogmans, 1982). We have investigated the possibility that cirsiliol interferes with calcium metabolism in smooth muscle cells. This interference could be exhibited by inhibition of Ca^{2+} influx, Ca^{2+} release from intracellular stores, Ca^{2+} binding to Ca^{2+} receptor proteins, or by combinations of these possibilities. Cirsiliol was found to inhibit, in concentration-dependent manner, the maximum contractions induced by Ach and to increase the EC_{50} of Ach. Since contractions induced by Ach depend mainly on calcium influx from the extracellular compartment through voltage-dependent and/or receptor-operated channels, and partially on calcium release from the intracellular stores (Devine *et al.*, 1972; Brading and Sneddon, 1980), it is suggested that cirsiliol might induce its effects by inhibiting Ca^{2+} influx and/or Ca^{2+} release from intracellular stores or might compete with Ca^{2+} to the Ca^{2+}-binding proteins in the ileal segments. Similar inhibition of Ach-induced contractions by flavones has been reported in the rat isolated ileum (Calixto *et al.*, 1986), and in the guinea-pig isolated ileum (Macander *et al.*, 1986; Abdalla *et al.*, 1988).

To test the effects of cirsiliol on Ca^{2+}-influx we incubated the ileum in a nominally Ca^{2+}-free depolarizing solution for 30 min then gradually increased Ca^{2+} concentration in the bathing solution. The increase in tension developed under such conditions is mostly due to the influx of Ca^{2+} from the extracellular compartment. Agents that depress the contractile responses in such type of experiment are thought to serve as Ca^{2+}-entry blockers (Godfraind, 1981). The observation that cirsiliol inhibited the contractile responses to $CaCl_2$ and increased the EC_{50} of $CaCl_2$ in a concentration-dependent manner indicates that cirsiliol inhibits Ca^{2+} influx from the extracellular compartment. This, however, does not rule out the possibility that cirsiliol enters the cell and competes with Ca^{2+} for Ca^{2+}-binding proteins.

To test the possibility that cirsiliol inhibits Ca^{2+} release from the intracellular stores, we incubated the ileum in a Ca^{2+}-free solution then we exposed it to various concentrations of cirsiliol. Since cirsiliol caused transient contractions in a nominally Ca^{2+}-free medium, it is suggested that the compound stimulates calcium release from intracellular stores, thus ruling out the possibility that the compound inhibits Ca^{2+} release from intracellular stores and the possibility that cirsiliol competes with Ca^{2+} for Ca^{2+} binding proteins.

The observation that cirsiliol potentiated the transient contractions induced in the ileum by 3×10^{-3} M Ach in Ca^{2+}-free solution when the latter was added just at the beginning of cirsiliol contraction and that cirsiliol inhibited the Ach-induced contractions when Ach was added after the cirsiliol contractions had faded out to the original baseline, gives further support to the suggestion that cirsiliol stimulates rather than inhibits Ca^{2+} release from intracellular stores. These observations further suggest that cirsiliol uses the same Ca^{2+} source used by acetylcholine so that when the Ca^{2+} source was totally depleted by high concentration of cirsiliol (Fig. 5IIC) no further contraction was induced by Ach and when the Ca^{2+} compartment was allowed to equilibrate in a Ca^{2+}-containing solution, Ach-induced contractions were restored (Fig. 5IIC). Further support to the conclusion that both cirsiliol and Ach utilize Ca^{2+} from the same compartment comes from the reverse experiment in which cirsiliol was added after Ach (Fig. 5III). Because the Ca^{2+} compartment has been depleted by Ach in such experiment, cirsiliol caused no further contraction. When the Ca^{2+} store has been refilled by washing with Ca^{2+}-containing solution, cirsiliol induced contractions were restored.

Flavonoids have been shown to possess inhibitory action on cyclic AMP phosphodiesterase (Petkov *et al.*, 1981; Nikaido *et al.*, 1982). Such inhibition, and the subsequent increase of cAMP, have been considered responsible for the myolytic activity observed with many flavonoids (Petkov *et al.*, 1983). Although some of the effects of cirsiliol observed in the present experiments can be explained as due to its effect on phosphodiesterase, the effect of the large concentrations of cirsiliol are unlikely attributable to such effect. This suggests that at such large concentrations cirsiliol possess multiple sites of action and its effects are nonspecific.

In conclusion, the flavone cirsiliol generally antagonizes the spasmodic effects induced in the ileal longitudinal segments by spasmogens that have different modes of actions. This antispasmodic effect may explain the claimed therapeutic effect of the source plant. Our observations are in support of the interference by cirsiliol with Ca^{2+} metabolism. Most likely, cirsiliol acts as a Ca^{2+} entry blocker inhibiting Ca^{2+} influx from the extracellular compartment. Furthermore, cirsiliol in high concentration (10^{-3}, 3×10^{-3} M) stimulates Ca^{2+} release from intracellular stores in the ileal smooth muscle.

Acknowledgements—This work is supported by a grant from the Deanship of Academic Research; University of Jordan.

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